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(54) Title: METHOD OF PRODUCING RECOMBINANT DIMERIC ENZYME

(57) Abstract

This invention relates to a method of producing a recombinant eukaryotic heterodimeric enzyme using a prokaryotic host. The method involves constructing a first DNA vector containing DNA encoding one of the subunits of the dimeric enzyme and then constructing a second DNA vector containing DNA encoding the second subunit of the enzyme. Once the DNA vectors are constructed, they are used to transform a prokaryotic host. The transformed prokaryotic host cell is then cultured under conditions appropriate for the expression of the dimeric enzyme. For example, using the method of the present invention, the heterodimeric isoform of creating kinase CKMB can be produced. This invention further relates to a method of producing a recombinant human dimeric enzyme in an active form using a prokaryotic host, the recombinant enzyme products produced using the method of the present invention, and a transformed prokaryotic host constructed by the method of the present invention.

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METHOD OF PRODUCING RECOMBINANT DIMERIC ENZYME

Background of the Invention

The analysis of creatine kinase (CK) isoforms is important for the early diagnosis of acute myocardial infarction (AMI) and for the early determination of coronary artery reperfusion in patients treated with thrombolytic therapy (Alan and Wu, Laboratory Medicine, 23(5):297-302, 1992). The CK isoforms CKMM and CKMB can be used to determine the success of reperfusion therapy, although measurement of MB isoforms provides the earliest and most definitive results. Levels of MM isoforms are also elevated in patients with skeletal muscle disease, and together with the relative MB index, can be useful for determining whether the muscle damage is acute or chronic.

Pure CKMB is needed for research studies of myocardial metabolism and the enzyme's catalytic mechanism and for preparation of standards and quality control materials for clinical analysis. Current methods of obtaining CKMB involve homogenizing heart tissue and precipitating the CKMB with ethanol or ammonium sulfate followed by ion exchange, gel filtration, and/or affinity chromatography (Grace and Roberts, Clin Chem Acta, 123:59-71, 1982; and Herman and Roberts, Anal Biochem, 106:244-252, 1980). These procedures involve multiple column-purification steps, are long and tedious, and may result in poor yields and low specific activities. In addition, the extracts of CKMB can contain large amounts of contaminants, such as albumin which co-fractionates and co-migrates with CKMB on chromatography and pathogens requiring special handling.

A need exists to improve and simplify the production of the different isoforms of CK to satisfy the requirements of researchers and clinicians.

Summary of the Invention

This invention relates to a method of producing a recombinant eukaryotic heterodimeric enzyme in an active form using a prokaryotic host. The method involves constructing a first DNA vector containing DNA encoding one of the subunits of the heterodimeric enzyme and, then constructing a second DNA vector containing DNA encoding the second subunit of the enzyme. Once the DNA vectors are constructed they are used to transform a prokaryotic host. The transformed prokaryotic host cell is then cultured under conditions appropriate for the expression of the heterodimeric enzyme. For example, using the method of the present invention, the heterodimeric iosform of creatine kinase, i.e., CKMB, can be produced.

This invention further relates a method of producing a recombinant human dimeric enzyme in an active form using a prokaryotic host.

Furthermore, the present invention relates to the recombinant enzyme products produced using the method of the present invention.

The invention further relates to a transformed prokaryotic host constructed by the method of the present invention.

Brief Description of the Drawing

Figure 1 a schematic illustration of the method of producing a recombinant dimeric enzyme of the present invention.

Detailed Description of the Invention

This invention is based upon the discovery that different isoforms of creatine kinase (i.e., CKBB, CKMM and CKMB) can be produced by constructing a DNA vector for the two different subunits of the enzyme and transforming a host cell with the two DNA vectors, the resulting transformed host cell being capable of expressing CKBB, CKMM and CKMB.

DNA vector

The term "DNA vector" is intended any replication competent vector which has the capability of having a DNA fragment inserted into it and, subsequently, the expression of that DNA insert by an appropriate host

cell. In addition, the DNA vector must be receptive to the insertion of a DNA fragment containing the DNA where the sequence encodes the subunits of the target eukaryotic dimeric enzyme such as creatine kinase (i.e., CKM and CKB). Furthermore, the DNA vector must contain a promoter which can be recognized by the host cell. Procedures for the construction of DNA vectors include those described in Maniatis *et al.*, Molecular Cloning, A Laboratory Manual, 2d, Cold Spring Harbor Laboratory Press (1989), herein referred to as Maniatis *et al.*.

The term "DNA fragment" is intended to encompass any DNA fragment that encodes an enzyme subunit. The DNA fragment once inserted into a DNA vector should be transmittable to a host microorganism by transformation or conjugation or transfection. Procedures for the construction or extraction of DNA fragments include those described in Maniatis *et al.* and others known by those skilled in the art.

Host

The transformed prokaryotic host of the present invention can be created by various methods by those skilled in the art. For example, transfection, transformation or electroporation as explained by Maniatis *et al.* can be used.

By the term "prokaryotic host" is intended any prokaryote capable of the uptake and expression of foreign DNA, i.e., DNA not originally a part of the prokaryotes's nuclear material. Suitable prokaryotes may include *Corynebacterium*, *Escherichia*, *Streptomyces* or *Bacillus*.

Recombinant Dimeric Enzyme

The recombinant dimeric enzyme of the present invention is intended to encompass any protein consisting of two subunits and possessing enzymatic properties.

The invention will be further illustrated by the following non-limiting Exemplification:

EXEMPLIFICATION

Materials and Methods:

Cloning CKMB cDNA using PCR

DNA fragments carrying cDNA encoding CKM and CKB proteins that had been cloned from a human cDNA library have been described (Perryman *et al.*, Biochem. and Biophys. Reasearch Comm., 140: 981-989, 1986; and Villarreal-Levy *et al.*, Biochem. and Biophys. Reasearch Comm., 144:1116-1127, 1987). We used PCR amplification to change the DNA sequence at the N- and C- termini to add restriction enzyme sites that were suitable for cloning the CK cDNA into Genzyme expression vectors. PCR primers were synthesized at Genzyme. The primers had the following 5' - 3' sequences;

SL22 (Nde1 site at the ATG start codon of CKB) GCC CAT ATG CCC TTC TCC AAC AGC CAC A

SL23 (EcoR1 site after the stop codon of CKB) GGA ATT CAT TTC TGG GCA GGC ATG AGG

SL24 (Nde1 site at the ATG start codon of CKM) GCC CAT ATG CCA TTC GGT AAC ACC CAC AAC

SL25 (BamH1 site after the stop codon of CKM) GCA GGA TCC TAC TTC TGG GCG GGG ATC AT.

The GeneAmp PCR Reagent Kit with AmpliTaq DNA Polymerase from Perkin Elmer Cetus (Norwalk, CT) was used for PCR reactions. The reactions were carried out following standard procedures outlined in the literature enclosed in the kit. Specifically, 5-30 ng DNA, 100 pmol primer DNA, 2.5 U AmpliTaq DNA polymerase, and 200 μmol dATP, dCTP, dGTP, dTTP were mixed with supplied buffer, and the reaction mix was overlayed with Ampliwax (Perkin Elmer Cetus). The PCR machine (Coy Laboratory Products, Inc. Grass Lake, MI) was programmed for a cycle of 94°C (melt) for 2 minutes, 55°C (anneal) for 2 minutes, 72°C (extend) for 2 minutes, this cycle was repeated 18 times. A final extension step was run for 10 minutes to allow for complete polymerization of all strands. PCR product (approximately 5-10 μg) was digested with Nde1 and EcoR1 (for CKB) or Nde1 and BamH1 (for CKM) and purified by electrophoresis through a 0.7% low melting point agarose TAE buffered gel (FMC BioProducts, Inc. Rockland, ME) for cloning into expression vectors. Electrophoresis was performed as described in Molecular Cloning (Sambrook, Fritsch and Maniatis, 1989. Cold Spring Harbor Press, Cold Spring Harbor, NY). Restriction

endonucleases were purchased from New England BioLabs (Beverly, MA) and digestion reactions set up as suggested by the manufacturer. DNA fragments were purified from gel slices using the Geneclean kit (Bio101, La Jolla, CA).

Constructing the Genzyme Expression Vector pRZ38

The expression vector was constructed at Genzyme as a derivative of the plasmid pBluescript SK +/- (available from Stratagene, La Jolla, CA). Expression is driven off the Lac promoter. Our vector, pRZ38, was constructed by adding an restriction enzyme site at the ATG start codon of the b-galactosidase gene through site-directed mutagenesis. Mutagenesis protocols were followed as described using the Muta-Gene In Vitro Mutagenesis Kit from BioRad (Richmond, CA). This change in the vector allows cloning of foreign genes after the LacZ promoter but maintains similar spacing from the promoter as in the native gene.

Cloning CKM and CKB DNA into pRZ38

Vector DNA (5 µg) was digested with Nde1 and EcoR1 (for CKB) or Nde1 and BamH1 (for CKM) and gel purified as above. Approximately 100 ng digested vector DNA and 100 ng digested PCR product were ligated in a 20 µl reaction (T4 DNA ligase purchased from NEB, Beverly, MA). Ligation reactions set up as described in Molecular Cloning. After overnight incubation at 15°C ligation mixes were diluted to 60µl with dH2O. 1µl of each was electroporated into electroporation competent E. coli strain MC1061. The Cell-Porator and Voltage Booster were purchased from Bethesda Research Labs (Bethesda, MD). Protocols for competent cell preparation and electroporation are described in the Instruction manual. Transformants were selected on LB agar plates supplemented with 50 µg/ml ampicillin. Transformants were analyzed for harboring the correct recombinant plasmid using the alkaline lysis miniprep technique (Molecular Cloning). Miniprep DNA was analyzed by restriction enzyme mapping. 25 ml cultures of pRZ52 (CKB) and pRZ53 (CKM) were grown in LB with 50µg/ml ampicillin and larger scale DNA preps were purified using QIAGEN plasmid purification columns (QIAGEN Corp., Chatsworth, CA). Dideoxynucleoside chain-termination DNA sequencing reactions

were carried out according to the standard protocols described in the Sequenase 2.0 kit from USB (Cleveland, OH). [-35S]-dATP (New England Nuclear) was used to radiolabel the sequences for visualization on Kodak XAR film. Reactions were separated through a 6% polyacrylamide gel, the gel dried, and exposed to film as described in Molecular Cloning. The DNA sequence representing the coding regions of CKB and CKM are in figure G1 and 2.

Once the CKM DNA sequence was determined to be correct the ampicillin resistance gene in pRZ53 was exchanged for the kanamycin resistance gene from Tn903 (Nomura *et al.*, Gene, 3:39-51, 1987). The ampicillin gene was cut out using the restriction enzymes Ssp1 and Bpm1, and the vector ends were blunt ended using T4 polymerase. The kanamycin resistance gene had been cloned into the polylinker in a pBR322 vector. The gene was cut out of the vector using BamH1 and was blunt ended with T4 polymerase. The resulting CKM plasmid is referred to as pRZ69.

Constructing the CKMB Co-expression Strain

pRZ52 and pRZ69 DNA was mixed together in a concentration of approximately 50 µg/ml and 1 µl of the mixture electroporated into 20 µl MC1061 cells. The cells were plated onto LB plates containing 50 µg/ml ampicillin and 50 µg/ml kanamycin to select for cells that had been co-transformed with both plasmids. Co-transformants were analyzed by restriction digestion of miniprep DNA (the standard technique is described in Molecular Cloning).

Analyzing Expression

Expression analysis of the co-transformants was carried out as follows. Clones were grown overnight in 2 ml LB, 0.2% glucose, 50 µg/ml kanamycin and 50 µg/ml ampicillin at 37°C shaking. In the morning 25 ml of LB with 50 µg/ml kanamycin and 50 µg/ml ampicillin was inoculated with 0.6 ml of the overnight culture and grown at 30°C shaking. Cultures were grown to A600 about 0.4 then sampled at one hour intervals for 4 hours. Cultures were left growing overnight and an additional sample taken. Whole cell samples were boiled in SDS-PAGE sample buffer and run through 12% polyacrylamide gels to assess protein production. Gels were obtained from BioRad, and protocols provided with gels were followed.

Several assays were used to determine the level and quality of the expressed recombinant protein. Cultures were grown as previously described. At A600 approximately 1.0 or after overnight incubation cells were harvested and resuspended in a lysis buffer (20 mM Bis-Tris pH 6.9, 0.25% Tween 20, 10 mM b-mercaptoethanol, 10 mM EDTA, 10 mM EGTA, 1mM PMSF). The resuspended cells were lysed by sonication on ice (or for larger scale analysis cells were lysed using the Microfluidizer from Microfluidics Corp., Newton, MA) and the cell debris removed by centrifugation. Samples were analyzed using the following assay systems after diluting into lysis buffer. The Creatine Kinase Reagent (Sigma 47-UV) is a spectrophotometric assay for kinetic determination of enzyme activity. The Creatine Phosphokinase (CPK) Isoenzymes Kit (Sigma 715-EP) separates the various isoforms (MM, MB, and BB) and stains for activity. The CK-MB assay system for the Abbott IMx analyzer uses a microparticle enzyme immunoassay (MEIA) to determine specific protein mass of CK-MB in a sample. (All protocols are provided with the assay kits.)

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ziegler, Robin J.
Long, Sue

(ii) TITLE OF INVENTION: Method of Producing Recombinant Dimeric Enzyme

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Bill Gosz, Esq., Genzyme Corporation
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(C) CITY: Cambridge
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) ZIP: 02139

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: William G Gosz
(B) REGISTRATION NUMBER: 27,787
(C) REFERENCE/DOCKET NUMBER: GEN3-10.0

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 252-7583
(B) TELEFAX: (617) 252-7600
(C) TELEX: 201223 GENCAMB

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1146 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCCCTTCT CCAACAGCCA CAACGCACTG AAGCTGCGCT TCCCGGCCGA GGACGAGTTC 60

CCCGACCTGA GCGCCCACAA CAACCACATG GCCAAGGTGC TGACCCCCGA GCTGTACGCG 120

GACGTGCGCG CCAAGAGCAC GCCGAGCGGC TTCACGCTGG ACGACGTCAT CCAGACAGGC 180

GTGGACAACC CGGGCCACCC GTACATCATG ACCGTGGGCT GCGTGGCGGG CGACGAGGAG 240

TCCTACGAAG TGTTCAAGGA TCTCTTCGAC CCCATCATCG AGGACCGGCA CCGGCGCTAC 300

AAGCCCAGCG ATGACGACAA GACCGACCTC AACCCCGACA ACCTGCAGGG CGGCGACGAC 360

CTGGACCCCA ACTACGTGCT GAGCTCGCGG GTGGCCACGG GCCGCAGCAT CCGTGGCTTC 420

TGCCTCCCCC CGCACTGCAG CCGCGGGGAG CGCCGAGCCA TCGAGAAGCT CGCGGTGGAA 480

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GCCCTGTCCA GCCTGGACGG CGACCTGGCG GGCCGATACT ACGCGCTCAA GAGCATGACG   540
GAGGCGGAGC AGCAGCAGCT CATCGACGAC CACTTCCTCT TCGACAAGCC CGTGTCGCCC   600
CTGCTGCTGG CCTCGGGCAT GGCCCGCGAC TGGCCCGACG CCGCGCGTAT CTGGCACAAT   660
GACAATAAGA CCTTCCTGGT GTGGGTCAAC GAGGAGGACC ACCTGCGGGT CATCTCCATG   720
CAGAAGGGGG GCAACATGAA GGAGGTGTTC ACCCGCTTCT GCACCGGCCT CACCCAGATT   780
GAAACTCTCT TCAAGTCTAA GGACTATGAG TTCATGTGGA ACCCTCACCT GGGCTACATC   840
CTCACCTGCC CATCCAACCT GGGCACCGGG CTGCGGGCAG GTGTCGATAT CAAGCTGCCC   900
AACCTGGGCA AGCATGAGAA GTTCTCGGAG GTGCTTAAGC GGCTGCGACT TCAGAAGCGA   960
GGCACAGGCG GTGTGGACAC GGCTGCGGTG GGCGGGGTCT TCGACGTCTC CAACGCTGAC  1020
CGCCTGGGCT TCTCAGAGGT GGAGCTGGTG CAGATGGTGG TGGACGGAGT GAAGCTGCTC  1080
ATCGAGATGG AACAGCGGCT GGAGCAGGGC CAGGCCATCG ACGACCTCAT GCCTGCCCAG  1140
AAATGA                                                             1146
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1146 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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ATGCCATTCG GTAACACCCA CAACAAGTTC AAGCTGAATT ACAAGCCTGA GGAGGAGTAC      60

CCCGACCTCA GCAAACATAA CAACCACATG GCCAAGGTAC TGACCCTTGA ACTCTACAAG     120

AAGCTGCGGG ACAAGGAGAT CCCATCTGGC TTCACTGTAG ACGATGTCAT CCAGACAGGA     180

GTGGACAACC CAGGTCACCC CTTCATCATG ACCGTGGGCT GCGTGGCTGG TGATGAGGAG     240

TCCTACGAAG TTTTCAAGGA ACTCTTTGAC CCCATCATCT CGGATCGCCA CGGGGGCTAC     300

AAACCCACTG ACAAGCACAA GACTGACCTC AACCATGAAA ACCTCAAGGG TGGAGACGAC     360

CTGGACCCCA ACTACGTGCT CAGCAGCCCG GTCCGCACTG GCCGCAGCAT CAAGGGCTAC     420

ACGTTGCCCC CACACTGCTC CCGTGGCGAG CGCCGGGCGG TGGAGAAGCT CTCTGTGGAA     480

GCTCTCAACA GCCTGACGGG CGAGTTCAAA GGGAAGTACT ACCCTCTGAA GAGCATGACG     540

GAGAAGGAGC AGCAGCAGCT CATCGATGAC CACTTCCAGT TCGACAAGCC CGTGTCCCCG     600

CTGCTGCTGG CCTCAGGCAT GGCCCGCCAC TGGCCCGACG CCCCTGGCAT CTGGCACAAT     660

GACAACAAGA GCTTCCTGGT GTGGGTGAAC GAGGAGGATC ACCTCCGGGT CATCTCCATG     720
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GAGAAGGGGG GCAACATGAA GGAGGTTTTC CGCCGCTTCT GCGTAGGGCT GCAGAAGATT 780

GAGGAGATCT TTAAGAAAGC TGGCCACCCC TTCATGTGGA ACCAGCACCT GGGCTACGTG 840

CTCACCTGCC CATCCAACCT GGGCACTGGG CTGCGTGGAG GCGTGCATGT GAAGCTGGCG 900

CACCTGAGCA AGCACCCCAA GTTCGAGGAG ATCCTCACCC GCCTGCGTCT GCAGAAGAGG 960

GGTACAGGTG CGGTGGACAC AGCTGCCGTG GGCTCAGTAT TTGACGTGTC CAACGCTGAT 1020

CGGCTGGGCT CGTCCGAAGT AGAACAGGTG CAGCTGGTGG TGGATGGTGT GAAGCTCATG 1080

GTGGAAATGG AGAAGAAGTT GGAGAAAGGC CAGTCCATCG ACGACATGAT CCCCGCCCAG 1140

AAGTAG 1146

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 381 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Met Pro Phe Ser Asn Ser His Asn Ala Leu Lys Leu Arg Phe Pro Ala
1               5                   10                  15

Glu Asp Glu Phe Pro Asp Leu Ser Ala His Asn Asn His Met Ala Lys
            20                  25                  30

Val Leu Thr Pro Glu Leu Tyr Ala Asp Val Arg Ala Lys Ser Thr Pro
        35                  40                  45

Ser Gly Phe Thr Leu Asp Asp Val Ile Gln Thr Gly Val Asp Asn Pro
    50                  55                  60

Gly His Pro Tyr Ile Met Thr Val Gly Cys Val Ala Gly Asp Glu Glu
65                  70                  75                  80

Ser Tyr Glu Val Phe Lys Asp Leu Phe Asp Pro Ile Ile Glu Asp Arg
                85                  90                  95

His Arg Arg Tyr Lys Pro Ser Asp Asp Asp Lys Thr Asp Leu Asn Pro
            100                 105                 110

Asp Asn Leu Gln Gly Gly Asp Asp Leu Asp Pro Asn Tyr Val Leu Ser
        115                 120                 125

Ser Arg Val Ala Thr Gly Arg Ser Ile Arg Gly Phe Cys Leu Pro Pro
    130                 135                 140
```

His Cys Ser Arg Gly Glu Arg Arg Ala Ile Glu Lys Leu Ala Val Glu
145 150 155 160

Ala Leu Ser Ser Leu Asp Gly Asp Leu Ala Gly Arg Tyr Tyr Ala Leu
165 170 175

Lys Ser Met Thr Glu Ala Glu Gln Gln Gln Leu Ile Asp Asp His Phe
180 185 190

Leu Phe Asp Lys Pro Val Ser Pro Leu Leu Leu Ala Ser Gly Met Ala
195 200 205

Arg Asp Trp Pro Asp Ala Ala Arg Ile Trp His Asn Asp Asn Lys Thr
210 215 220

Phe Leu Val Trp Val Asn Glu Glu Asp His Leu Arg Val Ile Ser Met
225 230 235 240

Gln Lys Gly Gly Asn Met Lys Glu Val Phe Thr Arg Phe Cys Thr Gly
245 250 255

Leu Thr Gln Ile Glu Thr Leu Phe Lys Ser Lys Asp Tyr Glu Phe Met
260 265 270

Trp Asn Pro His Leu Gly Tyr Ile Leu Thr Cys Pro Ser Asn Leu Gly
275 280 285

Thr Gly Leu Arg Ala Gly Val Asp Ile Lys Leu Pro Asn Leu Gly Lys
290 295 300

His Glu Lys Phe Ser Glu Val Leu Lys Arg Leu Arg Leu Gln Lys Arg
305 310 315 320

Gly Thr Gly Gly Val Asp Thr Ala Ala Val Gly Gly Val Phe Asp Val
325 330 335

Ser Asn Ala Asp Arg Leu Gly Phe Ser Glu Val Glu Leu Val Gln Met
340 345 350

Val Val Asp Gly Val Lys Leu Leu Ile Glu Met Glu Gln Arg Leu Glu
355 360 365

Gln Gly Gln Ala Ile Asp Asp Leu Met Pro Ala Gln Lys
370 375 380

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 381 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Pro Phe Gly Asn Thr His Asn Lys Phe Lys Leu Asn Tyr Lys Pro
1               5                   10                  15

Glu Glu Glu Tyr Pro Asp Leu Ser Lys His Asn Asn His Met Ala Lys
            20                  25                  30

Val Leu Thr Leu Glu Leu Tyr Lys Lys Leu Arg Asp Lys Glu Ile Pro
        35                  40                  45

Ser Gly Phe Thr Val Asp Asp Val Ile Gln Thr Gly Val Asp Asn Pro
    50                  55                  60

Gly His Pro Phe Ile Met Thr Val Gly Cys Val Ala Gly Asp Glu Glu
65                  70                  75                  80

Ser Tyr Glu Val Phe Lys Glu Leu Phe Asp Pro Ile Ile Ser Asp Arg
                85                  90                  95

His Gly Gly Tyr Lys Pro Thr Asp Lys His Lys Thr Asp Leu Asn His
            100                 105                 110

Glu Asn Leu Lys Gly Gly Asp Asp Leu Asp Pro Asn Tyr Val Leu Ser
        115                 120                 125

Ser Pro Val Arg Thr Gly Arg Ser Ile Lys Gly Tyr Thr Leu Pro Pro
    130                 135                 140
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His Cys Ser Arg Gly Glu Arg Arg Ala Val Glu Lys Leu Ser Val Glu
145 150 155 160

Ala Leu Asn Ser Leu Thr Gly Glu Phe Lys Gly Lys Tyr Tyr Pro Leu
165 170 175

Lys Ser Met Thr Glu Lys Glu Gln Gln Gln Leu Ile Asp Asp His Phe
180 185 190

Gln Phe Asp Lys Pro Val Ser Pro Leu Leu Leu Ala Ser Gly Met Ala
195 200 205

Arg His Trp Pro Asp Ala Pro Gly Ile Trp His Asn Asp Asn Lys Ser
210 215 220

Phe Leu Val Trp Val Asn Glu Glu Asp His Leu Arg Val Ile Ser Met
225 230 235 240

Glu Lys Gly Gly Asn Met Lys Glu Val Phe Arg Arg Phe Cys Val Gly
245 250 255

Leu Gln Lys Ile Glu Glu Ile Phe Lys Lys Ala Gly His Pro Phe Met
260 265 270

Trp Asn Gln His Leu Gly Tyr Val Leu Thr Cys Pro Ser Asn Leu Gly
275 280 285

Thr Gly Leu Arg Gly Gly Val His Val Lys Leu Ala His Leu Ser Lys
290 295 300

His Pro Lys Phe Glu Glu Ile Leu Thr Arg Leu Arg Leu Gln Lys Arg
305 310 315 320

Gly Thr Gly Ala Val Asp Thr Ala Ala Val Gly Ser Val Phe Asp Val
325 330 335

Ser Asn Ala Asp Arg Leu Gly Ser Ser Glu Val Glu Gln Val Gln Leu
340 345 350

Val Val Asp Gly Val Lys Leu Met Val Glu Met Glu Lys Lys Leu Glu
355 360 365

Lys Gly Gln Ser Ile Asp Asp Met Ile Pro Ala Gln Lys
370 375 380

CLAIMS

1. A method of producing an eukaryotic heterodimeric enzyme in an active form, comprising:
   (a) constructing:
      (i) a first DNA vector containing DNA encoding a first subunit of the enzyme; and
      (ii) a second DNA vector containing DNA encoding a second subunit of the enzyme;
   (b) transforming a prokaryotic host with:
      (i) the first DNA vector; and
      (ii) the second DNA vector; and
   (c) culturing the transformed prokaryotic host under conditions appropriate for the expression of the dimeric enzyme.

2. The method of Claim 1 wherein the eukaryotic heterodimeric enzyme is mammalian.

3. The method of Claim 1 wherein the eukaryotic heterodimeric enzyme is a kinase.

4. The method of Claim 3 wherein the kinase is a creatine kinase.

5. The method of Claim 1 wherein:
   (a) the first subunit is creatine kinase subunit B and second subunit is creatine kinase subunit M; or
   (b) the first subunit is creatine kinase subunit M and second subunit is creatine kinase subunit B.

6. The method of Claim 1 wherein the prokaryotic host is bacterial.

7. The method of Claim 6 wherein the bacterial host is a *Escherichia*.

8. A method of producing a human dimeric enzyme comprising:
   (a) constructing:
      (i) a first DNA vector containing DNA encoding a first subunit of the enzyme; and

(ii) second DNA vector containing DNA encoding a second subunit of the enzyme;

(b) transforming a prokaryotic host with:

(i) the first DNA vector; and

(ii) the second DNA vector; and

(c) culturing the transformed prokaryotic host under conditions appropriate for the expression of the heterodimeric enzyme.

9. The method of Claim 8 wherein the human dimeric enzyme is a kinase.

10. The method of Claim 9 wherein the kinase is creatine kinase.

11. The method of Claim 8 wherein the prokaryotic host is bacterial.

12. The method of Claim 11 wherein the bacterial host is a *Escherichia*.

13. The method of Claim 8 wherein the first and second subunit are creatine kinase subunit B.

14. The method of Claim 8 wherein the first and second subunit are creatine kinase subunit M.

15. A method of producing a creatine kinase enzyme comprising:

(a) constructing:

(i) a first DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:1; and

(ii) a second DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:2;

(b) transforming a bacterial host with:

(i) the first DNA vector; and

(ii) the second DNA vector; and

(c) culturing the transformed bacterial host under conditions appropriate for the expression of the dimeric enzyme.

16. A method of producing a creatine kinase enzyme comprising:
    (a) constructing:
        (i) a first DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:2; and
        (ii) a second DNA vector containing DNA encoding a DNA sequence selected from all or a portion of the DNA sequence of SEQ ID NO.:1;
    (b) transforming a bacterial host with:
        (i) the first DNA vector; and
        (ii) the second DNA vector; and
    (c) culturing the transformed bacterial host under conditions appropriate for the expression of the dimeric enzyme.

17. An eukaryotic heterodimeric enzyme produced by the method of Claim 1.

18. An human dimeric enzyme produced by the method of Claim 8.

19. A creatine kinase enzyme produced by the method of Claim 15.

20. A creatine kinase enzyme produced by the method of Claim 16.

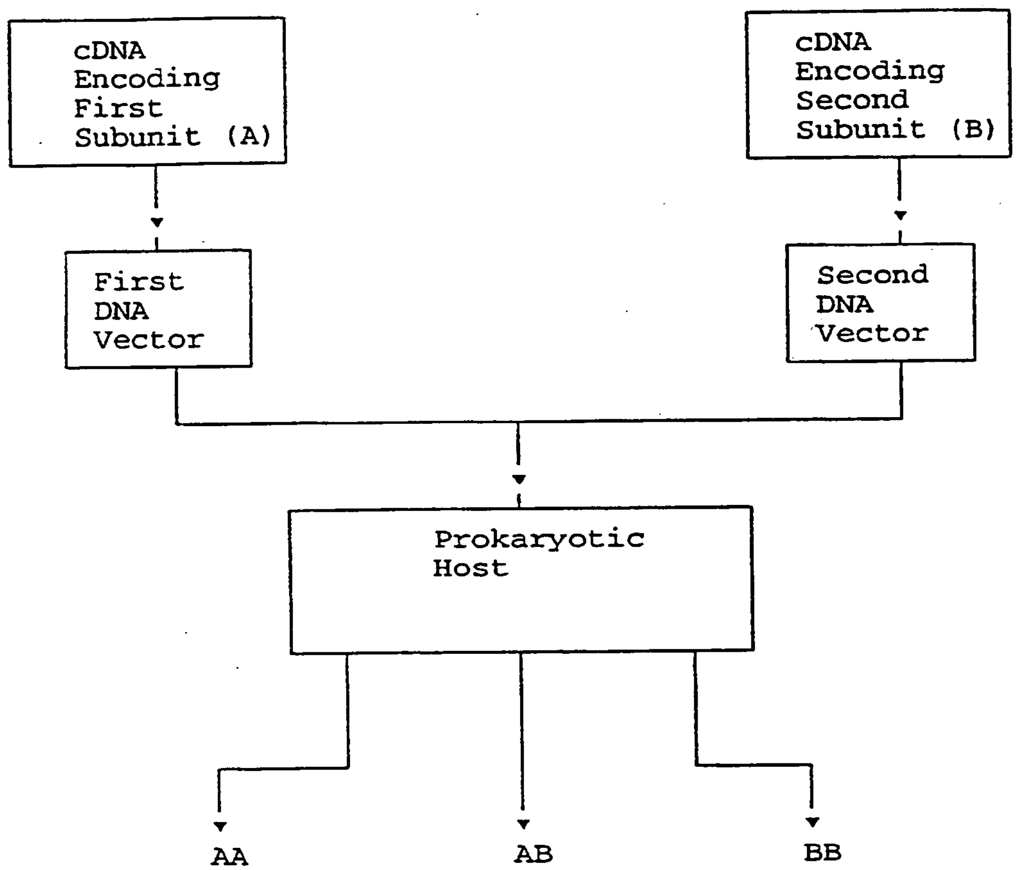
21. A transformed prokaryotic host produced by the method of Claim 1.

22. A transformed prokaryotic host produced by the method of Claim 8.

23. A transformed prokaryotic host produced by the method of Claim 15.

24. A transformed prokaryotic host produced by the method of Claim 16.

Figure 1


cDNA Encoding First Subunit (A)
cDNA Encoding Second Subunit (B)
First DNA Vector
Second DNA Vector
Prokaryotic Host
AA
AB
BB

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12624

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 1/21, 9/00, 9/12
US CL : 435/69.1, 183, 194, 252.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 183, 194, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog
search terms: creatine kinase, dimer?, heterodimer?, express?, coexpress?, recombinant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Vol. 267, No. 29, issued 15 October 1992, Mayer et al., "CDC43 and RAM2 encode the polypeptide subunits of a yeast type I protein geranylgeranyltransferase", pages 20589-20593, see the entire document.	1-24
Y	Journal of Biological Chemistry, Vol. 266, No. 18, issued 25 June 1991, Chen et al., "Cloning and expression of functional rabbit muscle creatine kinase in *Escherichia coli*", pages 12053-12057, see the entire document.	1-24
Y	Clinical Chemistry, Vol. 38, No. 6, issued 1992, Perryman et al., "Human recombinant creatine kinase isoenzyme and subform assay standards", page 981, see the entire document.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 DECEMBER 1994	JAN 30 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12624

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical Journal, Vol. 288, issued 1992, Furter et al., "Expression of active octameric chicken cardiac mitochondrial creatine kinase in *Escherichia coli*", pages 771-775, see the entire document.	1-24
Y	Clinical Chemistry, Vol. 39, No. 8, issued 1993, Friedman et al., "Recombinant creatine kinase proteins and proposed standards for creatine kinase isoenzyme and subform assays", pages 1598-1601, see the entire document.	1-24